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Distribution of Environmentally Regulated Genes of *Streptococcus suis* Serotype 2 among *S. suis* Serotypes and Other Organisms

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The occurrence of 36 environmentally regulated genes of *Streptococcus suis* strain 10 among all 35 *S. suis* serotypes was determined by using hybridization with the amplified genes as probes. In addition, the distribution of these genes among the virulence phenotypes of serotypes 1 and 2 was assessed. Hybridization was also performed with various other streptococcal species and nonstreptococcal bacterial species which may be present in pigs. Interestingly, probe ivs-25/iri-1, similar to *agrA* and *sapR*, hybridized only with *S. suis* serotype 1 and 2 strains with virulent phenotypes and is therefore suitable as a diagnostic parameter. Only one probe was specific for *S. suis*. This probe's sequence was identical to the *epf* gene, a putative virulence factor of *S. suis*. Probe ivs-31 was similar to a virulence factor of *S. suis*, namely, a gene encoding a fibronectin- and fibrinogen-binding protein. This probe hybridized only with oral streptococci. Nearly half of the probes (45%) hybridized with the oral streptococci (*S. oralis*, *S. milleri*, *S. sanguis*, *S. gordonii*, and *S. mitis*) and with *Streptococcus pneumoniae*. This indicates a close relationship between *S. suis*, the oral streptococci, and *S. pneumoniae* with respect to the selected environmentally regulated genes. One probe only hybridized with gram-negative species and therefore seems to be obtained by *S. suis* from a gram-negative organism by horizontal transfer.

Streptococcus suis is a major problem in the swine industry. *S. suis* causes a wide variety of infections in young piglets, including septicemia, meningitis, polyarthritis, and endocarditis (7, 22), and most often infected piglets do not survive. Occasionally, *S. suis* causes meningitis in humans (3). To undertake preventive health measures in the swine industry, it is important to recognize virulent bacteria and to distinguish such organisms from avirulent isolates from carriers. Adult pigs can carry *S. suis* in their noses and on their tonsils without exhibiting symptoms (2). From these adult carrier pigs, bacteria are transmitted to young pigs. Within pig herds, carrier rates of up to 100% have been described (8, 14). Despite this high carrier rate, the prevalence of disease in such herds is less than 5% (8, 14).

The discrepancy between the prevalences of carriership and disease may be related to the differences in virulence between *S. suis* strains and serotypes (21, 23, 24, 25). To date, 35 capsular serotypes have been described for *S. suis* (20), of which serotypes 1, 2, 7, 9, 14, and 1/2 are most frequently isolated from diseased pigs in Europe (27). Weakly virulent and avirulent phenotypes are found in serotype 2, and virulent and highly virulent phenotypes are found in serotype 1. These virulence phenotypes are differentiated by the expression of two proteins, muramidase-released protein (MRP) and extracellular factor (EF) (24). Recently, PCR tests were developed to detect serotypes 1, 2, 7, and 9 and to distinguish virulent serotype 2 strains from avirulent serotype 2 strains (19, 26, 28). For other serotypes, no reliable diagnostic methods are available.

Although there is a strong correlation between the expression of MRP and EF and the virulence of *S. suis* serotype 1 and 2 strains, MRP- and EF-negative *S. suis* strains still cause disease (18). Therefore, other factors must contribute to their virulence. The main goal of this study was to examine the possibility of using environmentally regulated genes for the development of diagnostic tools to detect virulent strains.

Since it is known that many important virulence factors are regulated and induced at specific stages of the infection process (13), we identified environmentally regulated genes of *S. suis* serotype 2 by using two different selection conditions (15). To do this, chromosomal DNA fragments of a pathogenic *S. suis* strain were cloned in a plasmid in front of a promoterless erythromycin resistance gene. The resulting plasmid library was introduced into a pathogenic *S. suis* strain. Subsequently, we selected *S. suis* clones in which erythromycin resistance had been induced under iron-restricted conditions and after infection of piglets with the library and treatment of the piglets with erythromycin. Infected animals developed specific signs of disease 3 to 8 days after infection. High numbers of bacteria were recovered from various tissues, and plasmid inserts were isolated from these recovered bacteria. Sequence analysis of these inserts revealed that a limited number of clones had been selected and that these clones were greatly enriched in the affected tissues (15). In total, 36 genes were identified as being environmentally regulated in *S. suis* serotype 2 (15); among these genes, regulatory genes, virulence factors, and metabolic genes were found.

We describe the presence and absence of the selected environmentally regulated genes in all 35 *S. suis* serotypes with

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TABLE 1. *S. suis* strains used

S. suis strain(s)	Serotype(s)	Presence of[a]:		Clinical source[b]	Virulence[c]	Source
		MRP	EF			
Reference strains	1–34 and 1/2					M. Gottschalk, Quebec, Canada
1313	1	−	−	Unknown	ND	Laboratory collection
1315	1	−	−	Unknown	ND	Laboratory collection
6555	1	−	−	Unknown	V	Laboratory collection
5637	1	−	−	Tonsil	V	Laboratory collection
6112	1	s	+	Organs	HV	Laboratory collection
6290	1	s	+	Meninges	HV	Laboratory collection
6388	1	s	+	Organs	HV	Laboratory collection
6436	1	s	+	Organs	ND	Laboratory collection
12	2	−	−	Tonsil	AV	Laboratory collection
16	2	−	−	Tonsil	AV	Laboratory collection
18	2	−	−	Tonsil	AV	Laboratory collection
T15	2	−	−	Tonsil	AV	Laboratory collection
17	2	+	L	Tonsil	WV	Laboratory collection
24	2	+	L	Human	WV	Laboratory collection
26	2	+	L	Human	ND	Laboratory collection
28	2	+	L	Human	WV	Laboratory collection
D282	2	+	+	CNS	V	Laboratory collection
3	2	+	+	CNS	V	Laboratory collection
10	2	+	+	Tonsil	V	Laboratory collection
22	2	+	+	Human	V	Laboratory collection

[a] −, absent; +, present; s, protein with a lower molecular mass is present; L, variant of protein with a higher molecular mass is present.
[b] CNS, central nervous system.
[c] HV, highly virulent; V, virulent; WV, weakly virulent; AV, avirulent; ND, virulence not determined. Data are from references 21, 23, and 24.

various virulence phenotypes and in various other streptococci and bacterial species which may be present in pigs. Based on these data, we found one gene, homologous to a gene in the database that encodes Agr, that could discriminate virulent serotype 1 and 2 strains from avirulent serotype 1 and 2 strains. This gene is very suitable for the development of a diagnostic test.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial isolates are listed in Tables 1 and 2. Streptococci were grown in Todd-Hewitt broth (Biotrading, Mijdrecht, The Netherlands) and plated on Columbia agar blood base plates (Biotrading) containing 6% (vol/vol) horse blood. *Actinobacillus pleuropneumoniae* was grown in brain heart infusion broth (BHI; Biotrading) plus 0.05% NAD (Fluka, Buchs, Switzerland) and plated on Columbia agar blood base plates (Biotrading) containing 5% heated sheep blood and 0.05% NAD (Fluka). *Haemophilus parasuis* was grown in BHI (Biotrading) and plated on Columbia agar blood base plates (Biotrading) containing 5% heated sheep blood. All other bacterial species were grown in BHI (Biotrading) and plated on heart infusion agar plates (Biotrading) containing 5% sheep blood. *Yersinia enterocolitica* was grown at a temperature of 30°C; all other species were grown at 37°C.

Chromosomal DNA isolation. Chromosomal DNA of the various bacterial species was isolated from 50-ml samples of stationary-phase growing cells. Bacterial cultures were centrifuged at 2,500 × *g* for 10 min. The cell pellet was resuspended in 5 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 10 mM disodium EDTA, 50 mM NaCl) containing 10 mg of lysozyme (Roche, Mannheim, Germany) per ml (or 10 μg of lysostaphin [Sigma, St. Louis, Mo.] per ml for *Staphylococcus aureus*) at 37°C for 10 min (or at 37°C for 30 min for *S. aureus*). Subsequently, 250 μl of 10% sodium dodecyl sulfate (SDS) and 40 μl of proteinase K (Merck, Darmstadt, Germany; 20 mg/ml) were added and the mixture was incubated at 70°C for 20 min (1 h for *S. aureus*). Suspensions containing the chromosomal DNA were extracted three to five times with equal volumes of phenol–chloroform–isoamyl ethanol (25:24:1) to remove proteins. Extracted DNA was incubated with 15 μl of DNase-free RNase A (Roche; 10 mg/ml) at 37°C for 15 min. Subsequently, chromosomal DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.4) and 2.5 volumes of 96% ethanol and then washed with an equal volume of 70% ethanol. The chromosomal DNA was dissolved in ultrapure water.

Radiolabeling of DNA probes. Probes for *ivs* and *iri* genes (ivs/iri probes) were amplified by PCR as described by Smith et al. (15) for the analysis of the genomic *S. suis* library in pIVS-E. GenBank accession numbers for the sequences of the probes are AF302190 to AF302207 for the *iri* gene probes and AF303226 to AF303247 for the *ivs* gene probes (15). The PCR products were purified by using the High Pure PCR product purification kit (Roche). Approximately 1 μg of DNA was radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol, 111 TBq/mmol; Amersham) by use of a random-primed labeling kit (Roche) as described by the manufacturers.

Detection of *ivs* and *iri* genes by dot blotting. One microgram of chromosomal DNA was spotted onto Genescreen Plus membranes. The membranes were incubated in 0.4 M NaOH–1 M NaCl at room temperature for 10 min to denature the DNA and incubated at room temperature for at least 10 min in 2× sodium chloride–sodium citrate (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]) for neutralization. The membranes were blocked in (pre)hybridization buffer (0.5 M sodium phosphate buffer [pH 7.2], 7% SDS, 1 mM EDTA) at 65°C for at least 30 min. Subsequently, the ^{32}P-radiolabeled probe was added to the membranes in 30 ml of hybridization buffer and the mixture was incubated overnight at 65°C. The membranes were washed twice with washing buffer 1 (40 mM sodium phosphate buffer [pH 7.2], 5% SDS, 1 mM EDTA) at 65°C for 30 min and twice with washing buffer 2 (40 mM $NaPO_4$ [pH 7.2], 1% SDS, 1 mM EDTA) at 65°C for 30 min. The signal was visualized with a STORM phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). As a positive control, a probe specific for *S. suis* 16S ribosomal DNA (rDNA) was used (17).

RESULTS

Distribution of *ivs* and *iri* gene sequences among all known *S. suis* serotypes. To examine the presence or absence of the selected *ivs* and *iri* genes in all 35 *S. suis* serotypes, we performed cross-hybridization experiments. The 16S rDNA positive control probe, as well as the probes for *iri-7* (similar to *rgpG*), *iri-8* (homologous to *gln* tRNA), *ivs-1* (similar to a transposase gene), and *ivs-29* (similar to a hypothetical gene), hybridized with all *S. suis* serotypes (Table 3). None of the probes reacted serotype specifically. Most probes hybridized with DNA of most serotypes, except those for *ivs-21* (*epf* gene),

TABLE 2. Bacterial strains used

Bacteria	Lancefield group or Gram stain result[a]	Source
Streptococci		
Streptococcus pyogenes	A	Laboratory collection
Streptococcus agalactiae	B	Laboratory collection
Streptococcus dysgalactiae	C	Laboratory collection
Streptococcus equi	C	Laboratory collection
Streptococcus equi subsp. *equisimilis*	C	Laboratory collection
Streptococcus equi subsp. *zooepidemicus*	C	Laboratory collection
Streptococcus bovis	D	Laboratory collection
Streptococcus uberis	E	Laboratory collection
Streptococcus milleri	F or G	Laboratory collection
Streptococcus group G	G	Laboratory collection
Streptococcus sanguis	H	Laboratory collection
Streptococcus group L	L	Laboratory collection
Lactococcus lactis subsp. *cremoris*	N	Laboratory collection
Streptococcus pneumoniae	None	Laboratory collection
Streptococcus mutans	None	B. Zaat, Amsterdam, The Netherlands
Streptococcus gordonii	None	B. Zaat, Amsterdam, The Netherlands
Streptococcus oralis	None	B. Zaat, Amsterdam, The Netherlands
Streptococcus mitis	None	B. Zaat, Amsterdam, The Netherlands
Other species		
Actinobacillus pleuropneumoniae	−	Laboratory collection
Haemophilus parasuis	−	Laboratory collection
Bordetella bronchiseptica	−	Laboratory collection
Campylobacter coli	−	Laboratory collection
Escherichia coli	−	Laboratory collection
Klebsiella pneumoniae	−	Laboratory collection
Pasteurella multocida	−	Laboratory collection
Proteus vulgaris	−	Laboratory collection
Pseudomonas aeruginosa	−	Laboratory collection
Salmonella enterica serovar Typhimurium	−	Laboratory collection
Yersinia enterocolitica	−	Laboratory collection
Bacillus subtillis	+	Laboratory collection
Enterococcus faecalis	+	Laboratory collection
Erysipelothrix rhusiopathiae	+	Laboratory collection
Listeria monocytogenes	+	Laboratory collection
Micrococcus	+	Laboratory collection
Staphylococcus aureus	+	Laboratory collection

[a] Lancefield group results are for streptococcal strains only.

ivs-25 and *iri-1* (similar to *agrA* and *sapR*), and *ivs-8* (similar to a transposase gene). Serotypes 32 and 34 did not hybridize with 27 probes. Also, for serotypes 33, 20, 22, and 26, limited hybridization was seen (Table 3). Probes ivs-21, ivs-25/iri-1, and ivs-8 hybridized with a limited number of serotypes (Table 3).

Distribution of *ivs* and *iri* genes among *S. suis* virulence phenotypes. We previously showed that serotype 1 and 2 strains differ in virulence (21, 23, 24, 25). Serotype 2 comprises virulent, weakly virulent, and avirulent strains; serotype 1 comprises highly virulent and virulent strains. The association of virulence with the occurrence of the various *ivs* and *iri* genes was studied. A 16S rDNA probe was used as a positive control. One probe, ivs-25/iri-1 (similar to *agrA* and *sapR*), discriminated between the virulence phenotypes of *S. suis* serotype 2 (data not shown). To confirm that there was a relationship between virulence and the presence of the *ivs-25* and *iri-1* genes, four strains of each virulence phenotype of serotype 1 and 2 were tested, where possible by using strains whose virulence had been tested in a pig model. The tests of four strains of each virulence phenotype invariably resulted in identical hybridization data. As shown in Table 4, all serotypes with virulence phenotypes hybridized with probe ivs-25/iri-1, while the avirulent serotype 2 strains did not hybridize. Therefore, probe ivs-25/iri-1 can be used to detect virulent *S. suis* strains among serotypes 1 and 2.

Hybridization of *ivs* and *iri* genes with various streptococci and other bacterial species. We next investigated the hybridization of the environmentally regulated genes with other bacterial species. The selected bacterial species from pigs included 17 streptococci besides *S. suis*, 6 additional gram-positive bacteria and 11 gram-negative bacteria. A 16S rDNA probe of *S. suis* serotype 2 containing the variable region as well as the conserved region that was used as a positive control showed a strongly positive signal in all cases. Probe iri-8 (homologous to *gln* tRNA) strongly hybridized with DNA of all streptococci and almost all other bacterial species (Tables 5 and 6). Apparently, *iri-8* is highly conserved among various bacterial species. Probes for a number of genes, namely, *ivs-21* (*epf* gene), *ivs-16* (similar to *atlR*), *iri-31* (*cps2A* gene), *iri-23* (similar to *yvyD*), *iri-11* (similar to *nrdD*), *iri-32* (similar to *ruvB*), *ivs-32* (similar to *fliF*), *ivs-36* (similar to *yqeG*), and *iri-13* (similar to MTCY336_33), did not hybridize to any of the other streptococci or to any of the other species tested, suggesting that these genes might be *S. suis* specific (Tables 5 and 6). However, except for *ivs-21* (*epf* gene), these genes showed homology to sequences in the database of bacteria present in our assay. Therefore, similar genes are present in bacterial species other than *S. suis*, and therefore the genes are not specific for *S. suis*. This finding indicates that the homology between the probes used and the genes present in the bacterial species was too low to detect the genes in the hybridization assay used and under the conditions applied. Probe ivs-21 (*epf* gene) hybridized only to *S. suis* and showed no homology in the database. Therefore, the *epf* gene is the only *S. suis*-specific gene that we found by using the selection procedures described above. Two probes, ivs-23/iri-24 (similar to *cpsY* and *oxyR*) and iri-16 (similar to *trmU*), hybridized to most of the streptococcal species but did not hybridize to other bacterial species except for *S. aureus*. Apparently, *ivs-23*, *iri-24*, and *iri-16* are conserved among the various streptococcal species. In contrast to other probes tested, ivs-2/iri-10 (similar to *yoaE*) hybridized to none of the streptococcal species except for *S. suis* but did hybridize with four gram-negative bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Typhimurium, and *Y. enterocolitica* (Tables 5 and 6).

DISCUSSION

In this study, we examined the distribution of 36 environmentally regulated genes of *S. suis* strain 10 among all 35 *S. suis* serotypes in order to improve detection of virulent *S. suis* strains. The probe ivs-25/iri-1 (similar to *agrA* and *sapR*) detected virulent and weakly virulent serotype 2 strains as well as virulent

TABLE 3. Distribution of ivs and iri genes among 35 reference strains of *S. suis* serotypes

Probe target(s) and functions	Database protein homolog(s)[a]	Hybridization result for indicated *S. suis* serotype[b]																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	1/2
Putative virulence factors																																				
ivs-21	EF	N	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	N	+	+	N	+	+	+	N	N	+	N	+	N	N	N	N	+
ivs-31	FlpA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+
Regulatory functions																																				
ivs-25, iri-1	SapR, AgrA	+	+	N	N	N	N	N	N	N	N	+	N	+	+	+	+	N	N	N	N	N	+	N	N	+	N	+	+	+	N	+	N	N	N	N
ivs-23, iri-24	CpsY, OxyR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+
ivs-16	AtlR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	N	+	+	+	N	N	N	+	+
ivs-20	AldR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	N	+	N	+	+
iri-31	Cps2A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	N	+	+	+	+	N	N	N	+	+
iri-23	YvyD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	+	+	+	+	+	N	+	N	+	+
Metabolic functions																																				
ivs-33	ThrC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	+	+	+	+	+	N	N	N	+	+
ivs-5	Tdk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+
ivs-18	NADH oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+
iri-11	NrdD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	+	+	+	+	+	+	+	N	N	N	+	+
iri-14	SulB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+
iri-7	RgpG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
iri-16	TrmU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+
iri-8	gln TrnA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
iri 32	RuvB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	N	+	N	+	+	+	N	+	+	+	+	+	N	N	N	+	+
iri-34	IlvA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	N	+	+	+	+	+	N	N	N	+	+
Transposases																																				
ivs-8	Transposase	+	+	+	+	+	+	+	N	+	+	+	N	+	+	+	+	+	+	N	N	N	+	N	+	N	N	+	N	+	N	+	N	+	+	+
ivs-1	Transposase 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Transporter functions																																				
ivs-2, iri-10	YoaE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	
ivs-3	OrfU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	
ivs-6, iri-2	YloD/ComYA/lviVI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	
Miscellaneous																																				
ivs-9	ComE ORF2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	
ivs-32	FliF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	N	N	N	+	
ivs-11	TabA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	
Unknown																																				
ivs-15	YdiB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	+	+	+	+	+	N	N	N	+	
ivs-34	YrrK	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	+	+	+	+	+	N	+	N	+	
ivs-36	YqeG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	
iri-13	MTCY336_33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	
iri-29	Yp15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	+	N	+	+	+	+	N	N	N	+	
ivs-19	Hypoth. prot.	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	
ivs-29	Hypoth. prot.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
iri-18	No homology	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+	+	N	+	
iri-4	No homology	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	
iri-3	No homology	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	N	+	
16S rDNA		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] See also reference 15. Hypoth. prot., hypothetical protein.
[b] +, positive hybridization signal; N, no hybridization signal.

TABLE 4. Distribution of *ivs-21*, *ivs-25*, and *iri-1* among different virulence phenotypes of *S. suis* serotypes 1 and 2

Probe target	Distribution among strains with the indicated virulence phenotype for[a]:				
	S. suis serotype 1		*S. suis* serotype 2		
	MRP[s] EF[+], HV	MRP[−] EF[−] V	MRP[+] EF[+] V	MRP[+] EF[+] WV	MRP[−] EF[−] AV
ivs-25 and *iri-1* (similar to *agrA* and *sapR*)	+	+	+	+	−
16S rDNA	+	+	+	+	+

[a] HV, highly virulent; V, virulent; AV, avirulent; MRP[s], protein of lower molecular mass is present. Four strains of each phenotype were tested and invariably resulted in identical hybridization patterns. −, absent; +, present.

and highly virulent serotype 1 strains. Since this probe hybridized only with virulent strains, it is suitable as a diagnostic parameter to detect virulent *S. suis* strains among serotypes 1 and 2. *ivs-25* was selected in vivo and *iri*-1 was induced under iron restriction (15). Both selected fragments are part of one gene in *S. suis* (unpublished results). This gene showed significant similarity to the *sapR* gene of *S. mutans* and to the *agrA* gene of *S. aureus*. In *S. aureus*, AgrA is a very strong regulator of several virulence factors (11). These data suggest that the *agrA* gene regulates transcription of genes that are important for virulence.

The data showed that one probe, ivs-21, was *S. suis* specific. Hybridization was not found between this probe and any of the other bacterial species used, and no homology was found in the database. This probe sequence was identical to the *epf* gene, a putative virulence factor of *S. suis* encoding EF, which was present in 25 of 35 *S. suis* serotypes. The ivs-21 probe did not react with serotypes 1, 16, 20, 22, 26, 27, 31, 32, 33, and 34. The ivs-21 probe hybridized with strains of *S. suis* serotypes 1 and 2 with all MRP and EF phenotypes.

Probe ivs-31 contained part of the fibronectin- and fibrinogen-binding protein gene of *S. suis* serotype 2. Its corresponding protein bound fibronectin and fibrinogen and was involved in the virulence of *S. suis* serotype 2 (10). This probe hybridized with four of the oral streptococci, *S. oralis*, *S. sanguis*, *S. gordonii*, and *S. mitis*. To date, the presence of such fibronectin- and fibrinogen-binding protein in *S. oralis*, *S. sanguis*, *S. gordonii*, and *S. mitis* has been unknown.

ivs-8, similar to a transposase gene, was found to be present in a small number of serotypes, including serotypes 1, 2, 7, 14, and 1/2. Serotype 9, another serotype often isolated from diseased piglets, did not have this transposase gene. Transposases may be involved in the insertion into the genome of foreign DNA-containing genes that are involved in virulence (9). Therefore, it might be very interesting to determine the genes flanking this transposase gene. The 5′ end of *ivs-8* shows homology to capsular genes of *S. pneumoniae*. For *S. suis*, the capsule is also an important virulence factor (16).

Probe ivs-9, similar to *comE* of *B. subtilis*, hybridized only to *S. suis*, *S. pneumoniae*, and to the oral streptococci, *S. oralis*, *S. milleri*, *S. sanguis*, *S. gordonii*, and *S. mitis*. Håvarstein et al. (12) described another competence gene that was present in the same streptococcal species. In *B. subtilis*, ComE is involved in competence development (1). For *S. pneumoniae*, it was shown that genes that are involved in competence are also involved in virulence (4). Although a natural transformation system has not been described for *S. suis*, the potential involvement of *ivs-9* in competence and in the pathogenesis of *S. suis* infections needs to be further investigated.

A number of genes, namely, ivs-21 (*epf* gene), ivs-16 (similar to *atlR*), iri-31 (*cps2A* gene), iri-23 (similar to *yvyD*), iri-11 (similar to *nrdD*), iri-32 (similar to *ruvB*), ivs-32 (similar to *fliF*), ivs-36 (similar to *yqeG*), and iri-13 (similar to MTCY336_33), hybridized only with *S. suis* DNA. Although these probes were *S. suis* specific, they showed similarity to sequences in the database of bacteria included in our assay. This means that the genes are not unique for *S. suis* but that the homology with other bacterial species was nonetheless too low to give a positive hybridization signal under the conditions used. Control hybridization experiments showed that fragments showing 75 to 80% homology on the DNA level will result in positive hybridization signals. Probes iri-3, iri-4, and iri-18 showed no similarity to DNA in the database, but all three probes hybridized with at least one other streptococcal species and therefore are also not *S. suis* specific. Probe iri-8, homologous to a *gln* tRNA gene, hybridized to almost all bacterial species tested, including tRNAs of many gram-negative organisms. tRNA genes are very conserved among bacterial species.

Most probes hybridized with the majority of the *S. suis* serotypes, except with serotypes 20, 22, 26, 32, 33, and 34. Chatellier et al. (6) and Brousseau et al. (5) determined the sequences of the 16S rDNA cluster and the chaperonin 60 gene, respectively, of all reference strains of *S. suis*. They showed that serotypes 20, 22, 26, 32, 33, and 34 were the most divergent serotypes.

Two other probes, ivs-23 and iri-16, hybridized with all 35 *S. suis* serotypes, with nearly all other streptococcal species, and with *S. aureus*. In the database, probe ivs-23 showed similarity to *cpsY* and *oxyR* of various streptococcal species. It is known that transcription regulators, such as *cpsY* and *oxyR*, are very conserved sequences. The other probe, iri-16, is similar to *trmU*, which encodes an RNA methyl transferase that is involved in the modification of nucleosides in bacterial tRNA. The function of this protein and its role in pathogenesis of infections are unknown. The fact that nearly all streptococci hybridized with the probe for *iri-16* indicates that this gene is highly conserved among streptococci.

Some probes showed hybridization only to *S. suis* and other streptococcal species. Apparently, such genes are highly conserved among streptococci. Interestingly, nearly half of the probes (45%) hybridized with the oral streptococci (*S. oralis*, *S. milleri*, *S. sanguis*, *S. gordonii*, and *S. mitis*) and *S. pneumoniae*. This indicates a close relationship between *S. suis*, the oral streptococci, and *S. pneumoniae* with respect to the selected environmentally regulated genes. Based on 16S rRNA sequencing, *S. suis* was most closely related to *S. bovis* and *S. equinus* (6).

Probe ivs-2 (similar to *yoaE*) hybridized with four gram-negative species, *E. coli*, *K. pneumoniae*, *S. enterica* serovar Typhimurium, and *Y. enterocolitica*. This gene had a considerable higher a G+C content than did the other selected *ivs* and *iri* genes (15), suggesting that the *yoaE* gene of *S. suis* was obtained by horizontal transfer from *E. coli* or another gram-negative organism.

In conclusion, the most promising candidate for improve-

TABLE 5. Hybridization of *ivs* and *iri* genes in several bacterial species

Probe target(s) and functions	Database protein homolog(s)[a]	Hybridization result for indicated bacterial species[b]																		
		S. equi	*L. lactis* subsp. *cremoris*	*S. mutans*	*S. bovis*	*S. agalactiae*	*S. equi* subsp. *zooepidemicus*	*S. equi* subsp. *equisimilis*	*Streptococcus* group G	*Streptococcus* group L	*S. pyogenes*	*S dysgalactiae*	*S. uberis*	*S. oralis*	*S. pneumoniae*	*S. milleri*	*S. sanguis*	*S. gordonii*	*S. mitis*	*S. suis*
Putative virulence factors																				
ivs-21	EF	N	N	N	N	N	N	N	N.	N	N	N	N	N	N	N	N	N	N	+
ivs-31	FlpA	N	N	N	N	N	N	N	N	N	N	N	N	W	N	N	W	W	W	+
Regulatory functions																				
ivs-25, iri-1	SapR and AgrA	N	N	N	W	W	W	+	+	+	N	N	N	N	N	N	N	N	N	+
ivs-23, iri-24	CpsY and OxyR	+	W	+	+	W	W	+	+	+	+	+	W	+	+	+	+	W	+	+
ivs-16	AtlR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-20	AldR	N	N	N	+	N	N	N	N	N	N	N	N	+	+	N	+	+	+	+
iri-31	Cps2A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-23	YvyD	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
Metabolic functions																				
ivs-33	ThrC	N	N	N	N	N	N	N	N	N	W	N	N	+	+	+	+	+	+	+
ivs-5	Tdk	N	N	N	W	W	W	+	+	N	+	+	+	+	N	W	W	W	W	+
ivs-18	NADH oxidase	N	N	N	N	N	W	N	W	N	W	+	N	N	N	N	N	N	N	+
iri-11	NrdD	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-14	SulB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	W	N	N	+
iri-7	RgpG	N	N	N	N	N	N	W	W	W	N	N	N	N	N	W	N	N	N	+
iri-16	TrmU	N	W	W	+	W	W	+	+	+	+	+	+	+	+	+	+	+	+	+
iri-8	Gln TrnA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
iri-32	RuvB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-34	IlvA	N	N	N	N	N	N	N	N	N	N	N	N	N	N	W	W	W	N	+
Transposases																				
ivs-8	Transposase	N	N	N	N	N	N	N	N	N	N	N	N	N	W	+	N	N	N	+
ivs-1	Transposase	N	N	W	N	N	+	N	N	N	N	+	N	+	+	+	+	+	N	+
Transporter functions																				
ivs-2, iri-10	YoaE	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-3	OrfU	N	N	N	N	N	N	W	W	N	N	W	W	N	N	N	N	N	N	+
ivs-6, iri-2	YloD/ComYA/lviVI	N	N	N	N	N	N	N	N	N	N	+	N	+	+	+	+	+	W	+
Miscellaneous																				
ivs-9	ComE ORF2	N	N	N	N	N	N	N	N	N	N	N	N	+	+	+	+	+	+	+
ivs-32	FliF	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-11	TabA	N	N	N	+	N	N	N	N	N	N	N	+	+	+	+	W	N	+	+
Unknown																				
ivs-15	YdiB	N	N	N	N	N	W	W	W	N	W	W	W	N	N	N	W	+	W	+
ivs-34	YrrK	N	N	N	N	W	N	+	+	+	+	+	+	W	N	+	+	+	+	+
ivs-36	YqeG	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-13	MTCY336_33	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-29	Yp15	N	N	N	N	+	+	+	+	+	N	+	N	+	+	N	N	N	N	+
ivs-19	Hypoth. prot.	N	N	N	N	N	N	N	N	N	N	N	N	N	N	W	N	N	N	+
ivs-29	Hypoth. prot.	N	N	N	+	W	N	W	W	N	W	W	N	+	+	+	+	+	W	+
iri-4	No homology	N	N	N	N	W	N	W	W	W	W	N	N	N	W	+	+	N	W	+
iri-18	No homology	N	N	N	N	N	N	N	N	N	N	N	N	N	N	W	N	N	N	+
iri-3	No homology	N	N	N	N	+	+	+	+	+	W	N	N	N	N	N	N	N	N	+
16S rDNA		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] See also reference 15. Hypoth. prot., hypothetical protein.

[b] +, positive hybridization signal; w, weak hybridization signal; N, no hybridization signal.

TABLE 6. Hybridization of *ivs* and *iri* genes in several gram-negative and -positive bacterial species

Probe target(s) and functions	Database protein homolog(s)[a]	Hybridization result for indicated bacterial species[b]																	
		Gram negative											Gram positive						
		Actinobacillus pleuropneumoniae	*Haemophilus parasuis*	*Bordetella bronchiseptica*	*Campylobacter coli*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pasteurella multocida*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Salmonella enterica* serovar typhimurium	*Yersinia enterocolitica*	*Enterococcus faecalis*	*Bacillus subtilis*	*Erysipelothrix rhusiopathiae*	*Listeria monocytogenes*	*Micrococcus*	*Streptococcus aureus*	*Streptococcus suis*
Putative virulence factors																			
ivs-21	EF	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-31	FlpA	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
Regulatory functions																			
ivs-25, iri-1	SapR/AgrA	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-23, iri-24	CpsY/OxyR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+	+
ivs-16	AtlR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-20	AldR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-31	Cps2A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-23	YvyD	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
Metabolic functions																			
ivs-33	ThrC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-5	Tdk	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-18	NADH oxidase	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-11	NrdD	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-14	SulB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-7	RgpG	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-16	TrmU	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	W	+
iri-8	Gln TrnA	N	+	N	+	+	+	+	W	N	W	W	+	+	W	+	N	+	+
iri-32	RuvB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-34	IlvA	N	N	N	N	N	N	N	N	N	N	N	N	+	N	N	N	N	+
Transposases																			
ivs-8	Transposase	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-1	Transposase	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
Transporter functions																			
ivs-2, iri-10	YoaE	N	N	N	N	+	W	N	N	N	W	W	N	N	N	N	N	N	+
ivs-3	OrfU	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-6, iri-2	YloD/ComYA/iviVl	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
Miscellaneous																			
ivs-9	ComE ORF2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-32	FliF	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-11	TabA	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
Unknown																			
ivs-15	YdiB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-34	YrrK	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-36	YqeG	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-13	MTCY336_33	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-29	Yp15	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-19	Hypoth. prot.	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
ivs-29	Hypoth. prot.	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-4	No homology	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-18	No homology	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
iri-3	No homology	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+
16S rDNA		+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	+	+

[a] See also reference 15. Hypoth. prot., hypothetical protein.

[b] +, positive hybridization signal; w, weak hybridization signal; N, no hybridization signal.

ment of *S. suis* diagnostics is probe ivs-25/iri-1, which encodes a putative general virulence regulator. It discriminates between virulent and avirulent serotype 1 and 2 strains. This probe can detect all virulent serotype 1 and 2 strains, while the present diagnostic methods are unable to detect virulent serotype 1 strains.

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